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Ecological Adaptation During Incipient Speciation Revealed by Precise Gene Replacement

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To understand the role of adaptation in speciation, one must characterize the ecologically relevant phenotypic effects of naturally occurring alleles at loci potentially causing reproductive isolation. The *desaturase2* gene of *Drosophila melanogaster* is such a locus. Two geographically differentiated *ds2* alleles underlie a pheromonal difference between the Zimbabwe and Cosmopolitan races. We used a site-directed gene replacement technique to introduce an allele of *ds2* from the Zimbabwe population into Cosmopolitan flies. We show that the Cosmopolitan allele confers resistance to cold as well as susceptibility to starvation when the entire genetic background is otherwise identical. We conclude that ecological adaptation likely accompanies sexual isolation between the two behavioral races of *D. melanogaster*.

It is often suggested that adaptation is the driving force behind divergence of populations leading to speciation (*1*–*3*). Population genetic and ecological data appear to support this view (*1*, *3*). However, establishing a causal link between a mutation, its effect on differential adaptation, and its effect, if any, on reproductive isolation has been difficult. A molecular genetic approach to this problem can be successful only if one is able to exchange naturally occurring alleles of candidate isolation genes between incipient species. Most important, the changes must involve only the gene under consideration, leaving the rest of the genome intact.

We focused on a naturally occurring DNA sequence polymorphism at the *desaturase2* (*ds2*) locus of *Drosophila melanogaster*. This locus encodes a Δ^9 fatty acid desaturase identified by mapping and association as the gene responsible for a cuticular hydrocarbon (CH) polymorphism between populations of this species (*4*–*6*). Hydrocarbons are mating pheromones in many insects (*7*, *8*), and differences in CH composition contribute to reproductive isolation between some *Drosophila* species (*9*, *10*). The functional $ds2^Z$ allele is found at high frequency in African and Caribbean populations. Females from these populations produce 5,9-heptacosadiene (5,9-HD) as the predominant CH. A 16-bp (base pair) deletion 5′ of *ds2* ($ds2^M$) results in apparent inactivation of the *ds2* gene (*5*, *6*). As a result, cuticles of Cosmopolitan females are rich in 7,11-heptacosadiene (7,11-HD) instead of 5,9-HD. The loss-of-function $ds2^M$ allele has spread throughout the rest of the world. Patterns of nucleotide diversity at the locus suggest that this spread may have occurred under the influence of positive selection (*6*).

In addition to its role in CH polymorphism, *ds2* is one of the candidate genes potentially responsible for strong premating isolation between females from Zimbabwe (Z) and males from Cosmopolitan (M) populations (*11*). These recently diverged populations may be incipient species (*12*–*14*). Whether *ds2* influences sexual isolation or not, the marked geographic pattern of *ds2* allele distribution has to be explained. We wanted to know whether some ecological forces are involved in the maintenance of this polymorphism and, if so, what those forces may be. Are different *ds2* alleles perhaps favored in different environments?

We can begin answering these questions by comparing the phenotypic and fitness effects of $ds2^M$ and $ds2^Z$ in a constant genetic background. However, conventional transgene technology, even in *D. melanogaster*, one of the most experimentally tractable model organisms, is not sufficient for the task. This approach does not allow targeting of the endogenous locus, and integration occurs at unpredictable sites in the genome. The influence of the surrounding chromosome environment on the expression of the transgene, as well as the effect of the insertion event itself on the neighboring genes, can yield a large variance in the expression of the trait being studied. This variability can greatly reduce, or even eliminate, the power to detect subtle differences in phenotype that are frequently of great importance in nature (*15*). The alternative approach based on backcross and introgression [e.g., (*16*, *17*)] does not offer the requisite control of genetic background.

Recent development of gene disruption in *D. melanogaster* by Rong and Golic (*18*, *19*) makes it possible to precisely manipulate any gene at its locus, thus eliminating most of the difficulties described above. We applied this technique in an attempt to shed light on the

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molecular and evolutionary mechanisms of incipient speciation between Z and M populations of *D. melanogaster*.

We made three types of constructs, as illustrated in Fig. 1 (*20*). Product 1 (Fig. 1A) is an engineered *ds2* allele shuttled in by P element, as is conventionally done. This construct is then mobilized through the FLP-*FRT* mechanism and reinserted into the native *ds2* locus, yielding Product 2 (Fig. 1A) (*19*). Product 2 represents the modification of the endogenous *ds2* gene and can assume either of the two forms: *ds2*[M, w^+, Z] (referred to as $ds2^{M,Z}$) or *ds2*[M, w^+, M] ($ds2^{M,M}$), where M denotes the nonfunctional M-type *ds2* allele, Z denotes the functional Z-type *ds2* allele, and w^+ is an eye-color marker allele from the *white* locus (*20*). Because the $ds2^M$ allele is probably a null, $ds2^{M,Z}$ contains only one functional copy of the *ds2* locus. To determine whether $ds2^{M,Z}$ behaves as a single-copy $ds2^Z$ allele, we included it in our assays. The $ds2^{M,M}$ allele served as a control for the duplication event itself as well as for w^+ marker insertion. The physical structure of these two forms is shown in Fig. 1B. Product 2 underwent "reduction," yielding Product 3, which again assumes either of two forms: $ds2^Z$ and $ds2^M$. The $ds2^Z$ form is the desired product: the change of the M-type *ds2* allele to Z-type, leaving the rest of the genome untouched. The $ds2^M$ form, the control, is the unchanged genotype, which went through the same procedure as $ds2^Z$ but remains unaltered (see Fig. 1C for structural details). All manipulations of the *ds2* locus were done in the M background.

We first assayed the cuticular hydrocarbon profile of the $ds2^Z$ and $ds2^M$ lines. The results are illustrated in Fig. 2, with an isofemale line from Zimbabwe for comparison. Although introduction of an insertion-type *ds2* allele significantly changes the 5,9-HD/7,11-HD ratio, the full Zimbabwe CH profile has not been recapitulated. It appears that the presence of the $ds2^Z$ allele is not sufficient to fully account for high levels of 5,9-HD despite the genetic mapping results and complete association between the *ds2* genotype and the CH phenotype (*6*). There likely exist minor loci whose presence was undetectable by either mapping or association. This highlights the limitations of either approach in revealing the full genetic architecture of complex traits.

The CH profile shown on Fig. 2 can be reproduced by the $ds2^{M,Z}$ and $ds2^{M,M}$ lines. In parallel with the construction of Product 1 (Fig. 1A), we also generated eight standard P element lines that carried $ds2^Z$ and checked their effect on CH composition. Transformant lines with insertions on both major autosomes as well as the X chromosome were isolated. Only two of these showed a noticeable change in the CH profile, indicating the importance of the location of the transgene insertion.

We next asked what ecological factors may be playing a role in the spread of the nonfunctional $ds2^M$ allele out of Africa, but not within Africa or the Caribbean. Levels of desaturation of lipids within membranes are known to influ-

Table 1. Introduction of $ds2^Z$ decreases cold tolerance. Cold tolerance was measured by transferring flies to the specified temperature, letting them recover, and counting the animals that did not survive. Flies were exposed to 4°C for 72 hours, to −1°C for 4.5 and 5 hours (the data were pooled), and to −10°C for 30 min. The *P* values were calculated with a 2 × 2 χ^2 test (*40*). [For details, see (*32*).]

Assay temperature	Line	Males			Females			Males and females		
		N_{dead}	N_{tot}	*P*	N_{dead}	N_{tot}	*P*	N_{dead}	N_{tot}	*P*
4°C, 72 h	$ds2^{M,Z}$	26	117	0.02	43	115	0.03	69	232	0.002
	$ds2^{M,M}$	12	118		28	118		40	236	
4°C, 72h	$ds2^Z$	27	124	0.074	23	116	0.16	50	240	0.017
	$ds2^M$	13	108		13	108		26	216	
−1°C, 4.5–5 h	$ds2^Z$	19	59	8×10^{-4}	29	58	2×10^{-5}	48	117	3×10^{-8}
	$ds2^M$	4	61		7	60		11	121	
−10°C, 0.5 h	$ds2^Z$	57	57	$<2 \times 10^{-16}$	50	65	$<2 \times 10^{-16}$	107	122	$<2 \times 10^{-16}$
	$ds2^M$	1	57		0	71		1	127	

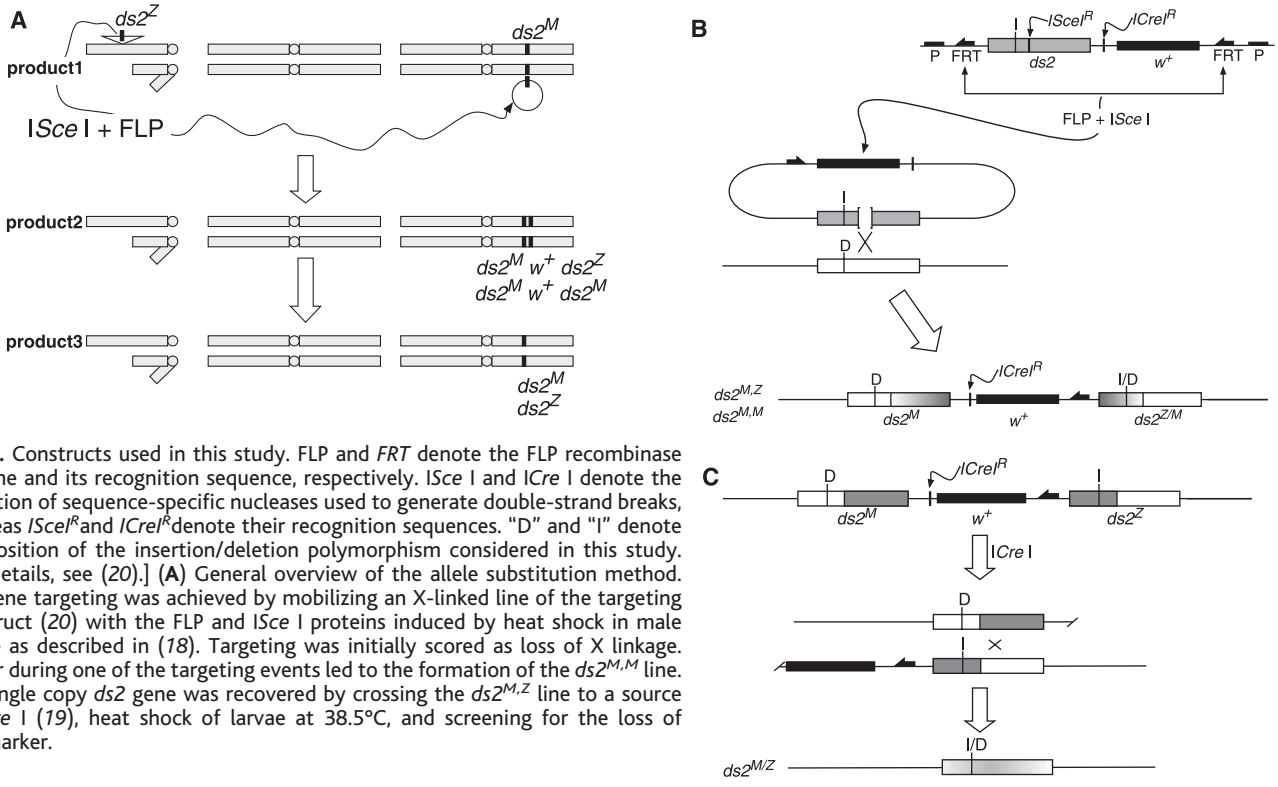

Fig. 1. Constructs used in this study. FLP and *FRT* denote the FLP recombinase enzyme and its recognition sequence, respectively. I*Sce* I and I*Cre* I denote the induction of sequence-specific nucleases used to generate double-strand breaks, whereas *ISceI*^{R}and *ICreI*^{R}denote their recognition sequences. "D" and "I" denote the position of the insertion/deletion polymorphism considered in this study. [For details, see (*20*).] (**A**) General overview of the allele substitution method. (**B**) Gene targeting was achieved by mobilizing an X-linked line of the targeting construct (*20*) with the FLP and I*Sce* I proteins induced by heat shock in male larvae as described in (*18*). Targeting was initially scored as loss of X linkage. Repair during one of the targeting events led to the formation of the $ds2^{M,M}$ line. (**C**) Single copy *ds2* gene was recovered by crossing the $ds2^{M,Z}$ line to a source of I*Cre* I (*19*), heat shock of larvae at 38.5°C, and screening for the loss of w^+ marker.

ence cold tolerance in many species, including *Drosophila* (*21*–*23*). Fatty acid desaturases have been shown to play a role in physiological adaptation to cold in various organisms through their influence on phospholipid composition (*22*, *24*). Comparison of cold tolerance within and between species of *Drosophila* (*25*–*29*) shows that animals captured in the tropics are more susceptible to cold than those caught in temperate climates.

Furthermore, in lepidoptera, fatty acid desaturases implicated in pheromone production and those potentially involved in cold resistance fall into two major phylogenetically distinct groups. Only one of these groups, which includes *ds2*, is found in *D. melanogaster* (*30*). Thus, *ds2* may play a role in both stress resistance and CH pheromone production (*30*). Finally, response to environmental stresses may have played an important role in the worldwide spread of *D. melanogaster* (*31*). It was thus logical to determine whether the transfer of the African $ds2^Z$ allele into a Cosmopolitan line would reduce its resistance to cold.

As shown in Table 1, cold tolerance decreases significantly as a result of the introduction of the $ds2^Z$ allele (*32*). This decrease is evident in both males and females under three different assay conditions, whereas the change in CH profile is manifested only in females. Our findings suggest that increased cold resistance may have helped the spread of the $ds2^M$ allele out of Africa.

In contrast to their susceptibility to cold, flies from tropical populations sometimes exhibit elevated resistance to starvation compared with those from temperate zones (*33*–*35*). Consequently, we were curious whether transfer of the African $ds2^Z$ allele would increase starvation tolerance, and this indeed turned out to be the case. The increase is highly significant in both sexes (Fig. 3, A to D), and both the "duplication" and "reduction" lines show a similar effect.

Additionally, CHs are thought to be involved in desiccation resistance (*36*). However, no consistent difference was observed between the $ds2^Z$ and $ds2^M$ lines (Fig. 3, E and F), although it is possible that we have not found the appropriate experimental conditions that would yield reproducible results.

Starvation tolerance in *D. melanogaster* often negatively correlates with body size [e.g., (*34*)], whereas cold tolerance shows the opposite correlation (*37*). Although these trends are by no means general, our results could be explained if the introduction of $ds2^Z$ makes flies smaller. We tested this possibility by measuring the influence of $ds2^Z$ on wing length, which tightly correlates with body size (*38*). No significant effect was observed in either males or females. For example, mean wing length of males from two $ds2^Z$

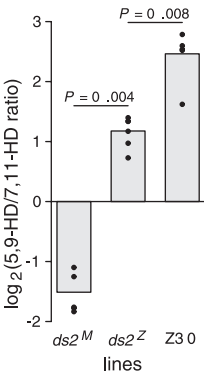

Fig. 2. Introduction of $ds2^Z$ changes the cuticular hydrocarbon profile. CH profiles were determined by gas chromatography as described in (*4*). 6-methyl-hexane was included with the 5,9-HD peak. The bars represent mean values and the dots each individual data point. The *P* values were calculated with the Wilcoxon rank-sum test (*40*). Z30 is an isofemale line from Zimbabwe.

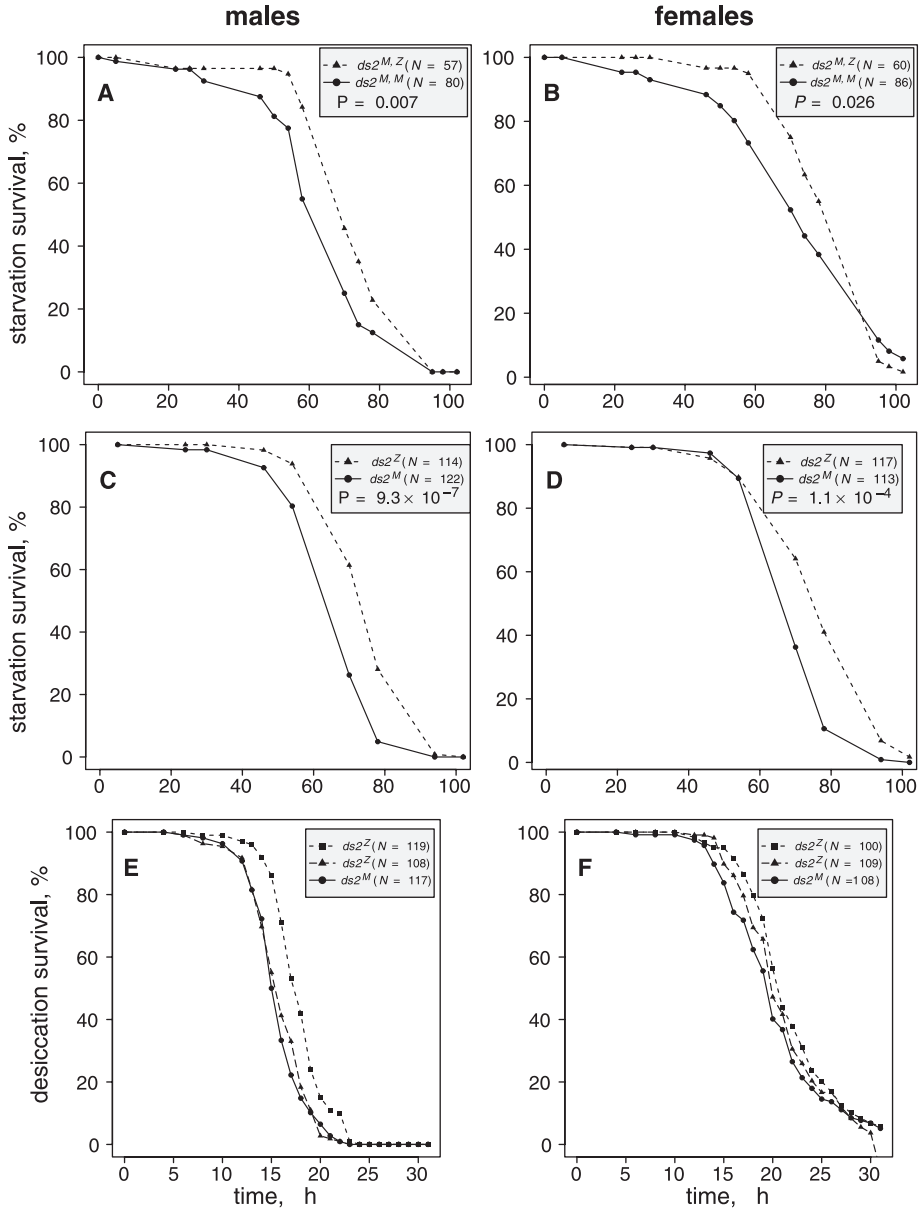

Fig. 3. Effect of $ds2^Z$ on starvation and desiccation tolerance. Dashed lines represent $ds2^Z$ or $ds2^{M,Z}$; solid lines represent $ds2^M$ or $ds2^{M,M}$. Numbers in parentheses indicate the total number of flies scored. *P* values were computed with the two-sample Kolmogorov-Smirnov test (*40*). Data for males are shown on the left, for females on the right. (**A** to **D**) Starvation survival of duplication [(A) and (B)] and reduction [(C) and (D)] lines was measured by transferring 3- to 4-day-old flies (*32*) into bottles with wet paper towels and no food. Dead flies were scored (*32*) every 4 or 16 hours. (**E** and **F**) Desiccation resistance of males (E) and females (F) was assayed by placing 3- to 4-day-old flies in Parafilm-sealed (Pachinery Plastic Packaging, Inc., Neenah, WI) bottles (*32*) with 2 g of Dri-Rite (Blue Island, IL). Dri-Rite was wrapped in Kimwipes (Kimberly-Clark Co., Rosewell, GA) to prevent direct exposure of flies. The survival curves were generated by counting the number of dead flies (*32*) every hour. The two dashed lines represent data for independently derived $ds2^Z$ stocks (*20*). None of the comparisons were significant (Kolmogorov-Smirnov $P > 0.05$) (*40*), with the exception of one of the $ds2^Z$ lines showing higher desiccation tolerance in males. This result was not reproducible, however, and was likely due to an aberration in fly-rearing conditions. [For details, see (*32*).]

lines was 1.23 and 1.26 mm, whereas it was 1.23 mm for the $ds2^M$ line (Wilcoxon rank-sum test $P > 0.1$ for all comparisons, sample sizes $N = 18$). If anything, one of the $ds2^Z$ lines shows an effect in the wrong direction.

Lack of sex specificity of the influence of *ds2* on both starvation and cold tolerance suggests that this effect is not due to differences in cuticular hydrocarbons per se. Perhaps it is due to an influence of *ds2* on phospholipid composition, as in many other organisms (*22*). Whatever the exact mechanism of *ds2* action, our results strongly suggest that it is involved in stress resistance. Note, however, that the $ds2^M$ allele appears to be the derived one (*6*). Consequently, we have restored the ancestral state at the *ds2* locus of the Cosmopolitan line, whereas the actual adaptation involved the loss of the $ds2^Z$ allele from the African population.

The possibility that ecologically driven adaptation at the *ds2* locus results in sexual isolation as a pleiotropic by-product is certainly intriguing. The role *ds2* may play in Z-M sexual isolation is being debated. The genetic basis of Z behavior is complex (*12*, *39*). Thus, *ds2* cannot be the only gene involved and, because the Caribbean flies carry the African $ds2^Z$ allele but exhibit M-type behavior, the locus has initially been excluded as a candidate sexual isolation gene (*4*). However, this lack of association across genetic backgrounds is inconclusive. A comparison within populations, in which the genetic background is randomized, is more informative. Indeed, when three African populations polymorphic for both Z behavior and *ds2* were tested, a positive correlation between the presence of $ds2^Z$ and the strength of female Z behavior was found in all of them (*11*). Thus, loss of $ds2^Z$ from the average African background may reduce Z-M sexual isolation.

Although the role of *ds2* in premating isolation remains to be firmly established, we have identified a potential ecological basis for the maintenance of pheromone polymorphism as a result of strong geographical differentiation at the *ds2* locus. Our ability to detect the role of *ds2* in differential adaptation depended crucially on manipulating the gene at its locus while leaving the rest of the genome intact. The phenotypic differences associated with *ds2* allele replacement are small enough to be drowned out by the noise introduced by the genetic background in conventional genetic analyses. Precise allele substitution thus promises to lead to insights into the molecular and evolutionary mechanism of adaptation and speciation.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/302/5651/1754/DC1

Materials and Methods

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The Needle Length of Bacterial Injectisomes Is Determined by a Molecular Ruler

Laure Journet, Céline Agrain, Petr Broz, Guy R. Cornelis*

Size determination represents a fundamental requirement for multicomponent biological structures. Some pathogenic bacteria possess a weapon derived from the flagellum. Like the flagellum, this type-III secretion apparatus, called the injectisome, has a transmembrane basal body, but the external component is a needle-like structure instead of a hook and a filament. Here, we provide evidence that the length of this needle is determined by the size of a protein, YscP, acting as a molecular ruler.

Yersinia pestis and *Y. enterocolitica*, the infectious agents of bubonic plague and gastroenteritis, respectively, share a common plasmid-encoded type-III secretion system consisting of the Ysc (Yop secretion) injectisome and the Yops (*Yersinia* outer proteins) that are secreted by this apparatus (*1*). The injectisome, made of 27 Ysc proteins, is thought to resemble those of *Salmonella enterica* and *Shigella flexneri*. These injectisomes, or "needle complexes," appear as two pairs of rings that are anchored to the inner and outer membranes of the bacterial envelope, joined by a central rod and supporting a hollow needle about 10 nm thick and 60 nm long (*2–4*). It is thought that the injectisome serves as a hollow conduit through which the secreted proteins travel across the two bacterial membranes and the peptidoglycan in one step.

Several Ysc proteins that are anchored in the inner membrane and form the core of the secretion apparatus are similar to proteins from the basal body of the flagellum, suggesting a common evolutionary origin (*5*). Not surprisingly, the *Salmonella* and *Shigella* injectisomes resemble the flagellar basal body (*6*) except that they are topped by a

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